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Anthocyanin Complex: Characterization and Cytotoxicity Studies

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***Abstract*—**Complexation of anthocyanins to mimic natural copigmentation process was investigated. Cyanidin-rich extracts from *Zea mays* L. *ceritina* Kulesh. and delphinidin-rich extracts from *Clitoria ternatea* L. were used to form 4 anthocyanin complexes, AC1, AC2, AC3 and AC4, in the presence of several polyphenols and a trace metal. Characterizations of the ACs were conducted by UV, FTIR, DSC/TGA and morphological observations. Bathochromic shifts of the UV spectra of 4 formulas of ACs were observed at peak wavelengths of about 510-620 nm by 10 nm suggesting complex formation. FTIR spectra of the ACs indicate shifts of peaks from 1,733 cm^{-1} to 1,696 cm^{-1} indicating interactions and a decrease in the peak areas within the wavenumber of 3,400-3,500 cm^{-1} indicating changes in hydrogen bonding. Thermal analysis of all of the ACs suggests increases in melting temperature after complexation. AC with the highest melting temperature was morphologically observed by SEM and TEM to be crystal-like particles within a range of 50 to 200 nm. Particle size analysis of the AC by laser diffraction gave a range of 50-600 nm, indicating aggregation. This AC was shown to have no cytotoxic effect on cultured HGEPp0.5 and HGF (all $p>$ 0.05) by MTT. Therefore, complexation of anthocyanins was simple and self-assembly process, potentially resulting in nanosized particles of anthocyanin complex.

***Keywords*—**Anthocyanins, complexation, purple corn cops, butterfly pea, physicochemical characteristics, cytotoxicity.

I. Introduction

ANTHOCYANINS are natural polyphenol pigments found in red, blue, and purple coloured plants which shown to be daily consumption fruits and vegetables (around 200 mg per person) in the U.S. [1]. Anthocyanins are also widely used as a natural supplements and have been shown to have anti-inflammatory activity [2], [3] anti-oxidant [4], [5] anti-carcinogenic [6] and anti-microbial including anti-candida activities [7], [8].

Cops of purple corns (*Zea mays* L., cv Zihei) are shown to be cyanidin-rich [9],[10] and blue flowers of butterfly peas (*Clitoria ternatea* L.) are delphinidin-rich [11]. Cyanidin and delphinidin are well reported to inhibit cyclooxygenases, TNF-α and NF-κB, promote wound formation and remodeling [12]-[15], inactivation of caspase and pro-apoptotic Bax proteins which lead to anti-apoptosis [16]-[19], and show progressive reducing oral lesion [20], [21]. Moreover, cyanidin and delphinidin was reported to have synergistic effect in anti-inflammation [3]. Thus, it is interesting in product development of these two different anthocyanins combination to be a potential candidate for oral inflammation in the future.

The use of anthocyanins in health products is limited due to physicochemically instable [22]-[26]. Anthocyanin co-pigmentation or complexation is a natural phenomenon which occurs when anthocyanins interact with neighbouring molecules, such as flavonoids, phenolic compounds or other anthocyanins [22]-[25]. The complex structure of anthocyanin copigments prevents their labile flavylium cation from the nucleophilic attack [22], [24], [26]-[31]. Stabilized anthocyanins are necessary for product development, and the methods used to provide anthocyanin complexes have not yet been well elaborated.

Complexation or copigmentaion can protect anthocyanins from degradation due to high pH or high temperature [22]-[24], [31]. Anthocyanins are prone to be degraded [24], [27], [32] particularly in aqueous solutions and basic conditions. Anthocyanin combined with flavonoids, phenolic acid and/or metals, has been reported and shown to retard degradation of anthocyanins [22]-[24], [31]. However, certain complexation such as metallo-complexation of anthocyanins could accelerate the irreversible formation of chalcone [30]. The stabilities of anthocyanin complex have been studied. Gris et al. studied anthocyanins extracted from Cabernet Sauvignon grape complex with caffeic acid and found that after forming complexation the half-life of anthocyanin were increased [28]. Moreover, Kunsági-Máté et al. revealed that metallo-complexation of malvidin-3-O-glucoside in a presence of ellagic acid and ferrous (II) ions help increasing activity energy (E_a) from 17.8 kJ/mol to 40.5 kJ/mol [30] which is similar to Zhang et al. that activity energy (E_a) of anthocyanins extracted from purple potato peel were increased after complexing with citric-acid monohydrate [31]. Thus, complexation of anthocyanins is a promising process to increase the stability of anthocyanins.

Cell culture model is a preliminary method purposing to screen toxicity of substances [12] which interacts directly between testing substances and living cells [13]. Oral inflammation, which occurs at gingival epithelial cells, is one of the most common conditions that found in all human being. A cultured gingival epithelial cell (HGEPp0.5), a primary human gingival epithelium cells that normally used in order to study the factors that effect to the cells, reported has pattern

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recognition receptors (PRRs) including Toll-like receptor [33], [34] which can be represent as the normal cells in oral cavity. Therefore, it is suitable to be tested the cell-response to the chemical substances. Another cell that related in inflammatory process, both immune and non-immune inflammatory reaction, is fibroblast [35]. Inflammation in periodontal disease arises on gingival fibroblast thus the use of culture human gingival fibroblast (HGF) is widely performed as *in vitro* study test on a substance [36]. Consequently, these two cells culture are selected to study the cytotoxicity effects of anthocyanin complex.

This study was aimed to screen complexation procedure for anthocyanins and select an anthocyanin complex based on the physicochemical characteristic for cytotoxicity in cultured cells representing human oral tissues. This provides a fundamental process to develop an anthocyanin complex for health product development.

II. Materials and Methods

A. Extraction and Complexation of Anthocyanins

Self-assembly complexation was used throughout the study. Dried blue butterfly pea petals (*Clitoria ternatea* L.) were purchased from a farm in Sisaket province in North-Eastern Thailand and were confirmed for identity using plant morphology [37]. To obtain the crude extract of petals of *C. ternatea* (CT), dried petals were ground into a fine powder using a blender, then soaked in boiling water for 30 min followed by freeze-drying overnights. Locally harvested dried cobs of purple waxy corn (*Zea mays* L. *ceritina* Kulesh.) from an open-pollinated variety (Kao Kum) were ground using a hammer mill to obtain corn cob powder. The corn cob crude extract (CC) was prepared by the same method as described for the flower petals.

TABLE I
Compositions for Comparative Screening of Anthocyanin Complex

Ingredients	AC1	AC2	AC3	AC4
Zea mays L. *ceritina* Kulesh. (CC)	5	5	5	7
Clitoria ternatea L. (CT)	2	2	5	2
Turmaricrhizome (Tur)	0.3	0.3	1	1
Caffeic acid	-	-	1	1
Piperine	0.3	0.3	-	0.1
Benzaldehyde	1	-	-	-
Zinc	1.5	1.5	0.1	0.2

An anthocyanin complex (AC) was formed using the previously described protocol [38]. In brief, the AC was formed by homogenizing aqueous extracts of CC, CT and other ingredients (ratio followed Table I) in 80°C water bath for 30 min. The mixture was placed at -20°C overnight before defrosting at room temperature to allow precipitation of solids. The precipitate was collected and oven dried to obtain each AC powder which was stored at 25°C in light-protected containers until use.

B. Screening Tests

Shifts in maximum wavelengths of the UV spectrophotometry have been introduced to monitor complexation of anthocyanins [22], [24], [39]. AC1, AC2, AC3 and AC4 were diluted with deionized water to the same concentration of 1 mg/ml and subjected to spectral record by a UV-visible spectrophotometer (1240 model, Shimadzu, Tokyo, Japan).

Fourier transform infrared spectroscopy (FTIR) was used to determine the fingerprint and molecular structure of the extracts and complex. Dry samples were mixed with potassium bromide (KBr) using the KBr technique with a ratio of 1:150 mg (sample: KBr) with 10 ton of hydraulic pressure. IR spectra were recorded on FTIR spectrometer (PerkinElmer Inc., Spectrum One program, Massachusetts, USA) in the region from 4000–400 cm^{-1}.

Thermal analysis to characterize the effect of complexation on thermal properties of the AC samples was conducted by differential scanning calorimetry (DSC) for determinations of melting points or transition temperatures (exothermic or endothermic) and thermal gravimetric analysis (TGA) to monitor weight loss by heat. Samples (3-5 mg each) of CC, CT and ACs were placed in a pierced aluminium pans and heated at a scanning rate of 5°C/min from 25°C to 200°C under air atmosphere using blank aluminum pan as a reference by DSC (DSC_{822}^e, Mettler Toledo, U.S.A) and TGA ($TGA/SDTA_{851}^e$, Mettler Toledo, U.S.A) with cooling machine in a nitrogen bath (40 ml/min).

C. Cytotoxicity of a Selected AC

Since all of the ACs showed some changes in UV and FTIR shifts, the selection of AC for characterization was conducted in accordance to its best performance from thermal analysis [31]. The selected AC was subjected to cytotoxicity test with explanation by solubility determination and morphological observations.

Cytotoxicity

Human Gingival Epithelial Progenitors, Pooled: HGEPp.05 (Cellntec®, Bern, Switzerland) were cultured in 75-cm^2 tissue culture flasks in Keratinocyte-SFM medium (Invitrogen, U.S.A.) while Human Gingival Fibroblast cells: HGF-1 (ATCC® CRL-2014™) was cultured in 20% high premium FBS (Gibco Inc., U.S.A.) and 80% DMEM (Gibco Inc., U.S.A.). Both cells culture medium were supplemented and 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 25 µg/ml of amphotericin B (Gibco Inc., U.S.A.), and kept in a humidified CO_2 incubator (Shell Lab, U.S.A.) containing 5% CO_2 controlled at 37ºC.

When cells growth reached its plateau which take about 7-10 days, the cells were trypsinized by 0.25% trypsin/EDTA (Gibco Inc., U.S.A.) solution for 5 min, fresh culture medium would use to inactivate the trypsin, re-suspended the cells palette again, and then seeded cells 100 µl of cells suspension in concentration of 10,000 cells/well into a 96-well plate, and incubated 24 h prior to use.

All AC concentrations were dissolved in free FBS culture medium. Negative control was the free FBS culture medium and positive control was 10%hydrogen peroxide (H_2O_2; Vidhyasom co., Ltd., Thailand). Cell viability (%) was estimated by comparison to the negative control (media). Then, the % cell viability was calculated as the following equation:

$$\% \text{ viability} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of negative control}} \times 100$$

Solubility

It was observed that the selected AC was not as highly water soluble as its anthocyanin sources from CC and CT. To aid dissolution of AC for cytotoxicity study, solubility of the AC was carried out in 9 types of solvents, i.e. deionized water (at pH 1, 7, and 12), methanol (Vidhyasom, Thailand), ethanol (Vidhyasom, Thailand), hexane (Sigma-Aldrich, U.S.A.), chloroform (Sigma-Aldrich, U.S.A.), dimethyl sulfoxide (DMSO; Sigma-Aldrich, U.S.A.), polyethylene glycol (PEG) 400 (Vidhyasom, Thailand).

Particle Size Analysis

Since complexation tends to enlarge the molecular size of the complexing compounds, and, it may be a burden to cytotoxicity study if the size of the AC was enlarged to some extent. Thus, particle size of the AC was investigated by laser diffraction using a Zetasizer (Malvern Instruments, Worcestershire, UK). Samples of AC were diluted with filtered purified water, through 0.2 μm cellulose acetate filters (Sartorius Stedim Biotech, Germany), and filled in polystyrene cuvettes for repeated reading. Six replicates were conducted for each sample.

Morphology

To confirm with particle size analysis and to observe the morphology of the selected AC using scanning electron microscope (SEM, LEO 1450 VP, Germany), a trace amount of the sample was mounted, attached on a stub, coated with gold and observed at a magnification of 2,000-10,000. Transmission electron microscopy (TEM, FEI Company, U.S.A.) was used to determine the microstructures of samples of the AC. The samples were diluted as appropriated with ultrapure water before preparation for TEM. A drop of each sample was applied to a copper grid coated with carbon film. The resultant was air-dried and subsequently observed under TEM at a magnification of 20,000-10,000.

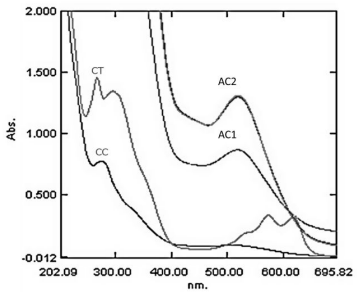
D. Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Coefficients of variation (CV) were calculated for determination of precision of data and methods. Statistical significance was determined by one-way analysis of variance (ANOVA). The significant level was considered at $p< 0.05$.

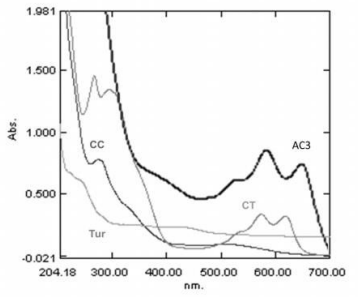
III. Results and Discussion

The colour of aqueous extracts of blue petals of *Clitoria ternatea* L. is dark blue solution while purple cobs of *Zea mays* L. *ceritina* Kulesh.is dark red-blue solution. After forming anthocyanin complex, all AC formula solutions were similarly dark purple but different precipitates which change with pH.

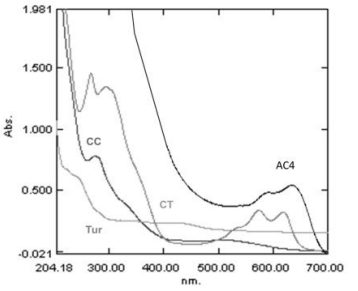
UV spectra were mainly focused on the region of 500 -700 nm and revealed bathochromic shifts as a results of complexation in comparison to its anthocyanin sources, CC and CT, as shown in Fig. 1 and Table II. At the same concentration 1 mg/ml, CC extract gave maximum UV absorption at a wavelength of 508 nm while CT extract exhibits 2 peaks at wavelengths of 574 and 618 nm.



(a) AC1 and AC2



(b) AC3



(c) AC4

Fig. 1 UV spectrum of the *Zea mays* L. *ceritina* Kulesh. (CC) and *Clitoriaternatea*L. (CT) comparing to the UV spectrum of (a) AC1 and AC2, (b) AC3 and (c) AC4

Table II summarizes the peak shifts ofAC1 and AC2 from 508 to 518.5 ($\Delta\lambda$ = 10.5) nm, AC3 from 508 to 576 ($\Delta\lambda$ = 78) nm, 574 to 586 ($\Delta\lambda$ = 12) nm and 618 to 628 ($\Delta\lambda$ = 10) nm,

while AC4 was from508 to 580 (Δλ = 72) nm, 574 to 580 (Δλ = 6) nm and 618 to 621 (Δλ = 3) nm. From the UV-visible spectroscopy, all AC formula were observed the bathochromic shifts mainly at 508 nm indicating complex formation.

AC1 and AC2 with 2.5 times higher CC (λ_{max} 508 nm), than CT (λ_{max} 574 and 618 nm) could affect the overall peak of 518 nm. Benzaldehyde in AC1 [40] for comparison to non–benzaldehyde in AC2 affected the absorbance but not the bathochromic shift. This indicates that CC and CT anthocyanins play vital roles in the shifts. In AC3, 1:1 ratio of CC and CT provided the shift in all peak of anthocyanin-rich ingredients and observed the bathochromic shifts in all wavelengths. AC4 contained 3.5times higher CC than CT giving higher bathochromic shifts towards CC.

TABLE II
UV ABSORBANCE OF ANTHOCYANIN COMPLEX AND ITS COMPONENTS

Samples	λ_{max} (nm)	Absorbance (A)	Δλ (nm)		
			CC 508nm	CT 574nm	CT 618nm
Zea mays L. *ceritina* Kulesh.(CC)	508	0.92	-	-	-
Clitoria ternatea L. (CT)	574	0.336			
	618	0.322			
AC1	518.5	0.8	10.5	-	-
AC2	518.5	1.298			
AC3	586	0.844	78.0	12.0	10.0
	628	0.762			
AC4	580	0.528	72.0	6.0	3.0
	621	0.561			

CC and CT were shown to be complexed in the presence of other components with differences in colours and UV absorbance shifts in related to the ratios of CC and CT.

The infrared spectra, as shown in Fig. 2 (a), of the CC and CT extraction revealed that the hydrogen group was formed (bands around 3,400-3,500 cm^{-1}). After forming the AC (all formula), this bands (3,400-3,500 cm^{-1}) was decrease but showed the greater area of oxonium ion molecules (band below 3,000 cm^{-1}). Moreover, AC3 and AC4 formula showed the peak shift, shown in Fig. 2 (b), from the area between 1,600 cm^{-1} to 1,640 $^{-1}$ which indicated the –C=C– stretch alkenes and/or the benzene rings. The strengths of the hydrogen bonding related with thermodynamic properties of the complex reaction. Around the band 1,700 cm^{-1} which represent the C=O stretch (α, β–unsaturated of aldehydes, ketones). Therefore, the changes in peak shift and peak area of AC comparing to CC and CT suggested that the interaction of functional groups were occurred after forming complexation.

TABLE III
SUMMARY OF THE THERMAL ANALYSIS OF ALL ANTHOCYANIN COMPLEX FORMULA

Samples	calories (mJ)	onset (°C)	% residue	Melting temperature (°C)
CC	4,401.78	136.83	78.22	138.03
CT	559.16	150.59	65.84	152.32
AC1	11.35	153.70	79.19	154.21
AC2	824.78	160.33	83.74	160.90
AC3	24.24	166.78	80.95	167.71
AC4	179.00	155.82	-	165.70

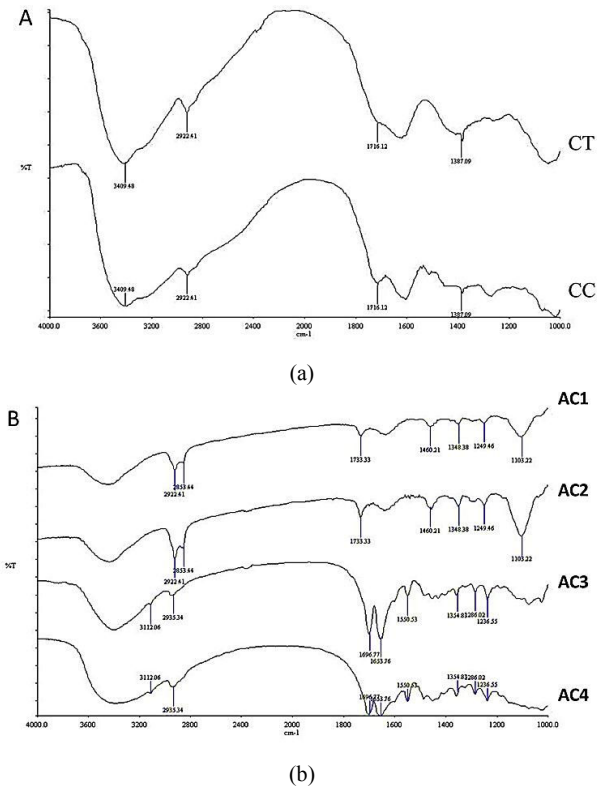


Fig. 2 FTIR spectrum of the (a) *Zea mays* L. *ceritina* Kulesh. (CC) and *Clitoria ternatea* L. (CT) and (b) anthocyanin complex (ACs)

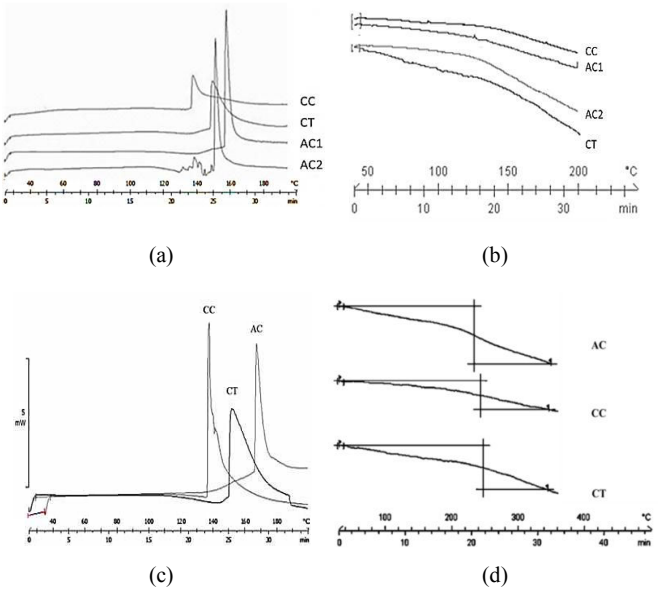
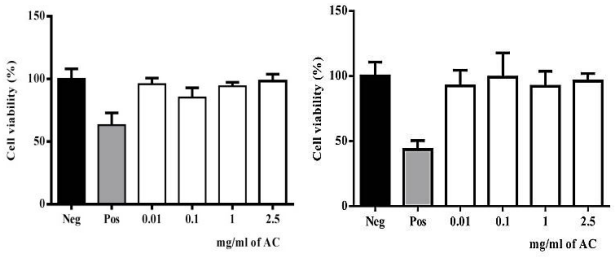


Fig. 3 Thermographs of *Zea mays* L. *ceritina* Kulesh. (CC) and *Clitoria ternatea* L. (CT)comparing to anthocyanin complex (ACs) as DSC thermographs of (a) AC1 and AC2, (b) TGA thermographs of AC1 and AC2, (c)DSC thermographs of AC3 and (d) TGA thermographs of AC3 under nitrogen flow

The effects of the complexation on the thermodynamics of anthocyanin complex are evaluated by their transition temperatures in solid state. The DSC thermogram for CC and CT shows a strong edothermic effect while reduced this effect after forming AC as shown in Fig. 3 and Table III.

The phase behavior and the degradation of CC and CT started at 136.8 and 150.6°C, respectively, while of AC1, AC2, AC3 and AC4 at 153.7, 160.33, 166.8 and 155.82°C, respectively, which related to the lower thermooxidative degradation after forming complex. The melting points of all AC formula provided increased higher melting point value than the extracts. AC3 was shown to provide highest phase behaviour and increased melting temperature. At a higher temperature, about 200°C, the exothermic signal of degradation is associated to the mass loss of this sample due to thermooxidation, as recorded in TGA which observed that the AC provided higher % residual (mass) than the extracts. This exothermic signal in DSC is associated with a decrease of heat capacity of the sample due to mass loss. The TGA revealed that at the equal temperature increased, the amount of residue AC after increasing the heat was also higher than the extraction but %residue is higher than both extracts as shown in Fig. 3. These results of all AC formula were similar to [31] which showed the shift of peaks in DSC profile but different in the diagram, our reported the endothermic state while [31] reported both endothermic and exothermic state which may cause from the different of forming complex molecules.

From these results, AC3 showed better characterization and physicochemical properties over than others as showing high melting point and high %residual after exposed to the heat which could be related to the high stability over than other [31]. According to the characterization, physicochemical properties, and thermal analysis, AC3 was selected to be used for further study.



(a) HGEP p0.5 cells (b) HGF cells

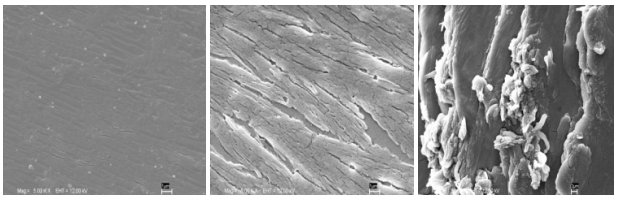
Fig. 4 Mean viability (%) of HGEPp0.5 cells (a) and HGF cells (b) treated with 0.01-2.5 mg/ml of AC for 24 h. Negative control (Neg) using medium solution for its relevant blanks, Positive control (Ps) using 10%H_2O_2 solution

HGEP p0.5, representing normal oral epithelium [33], [34], and HGF, representing gingival fibroblasts [35], [36], exposed to AC concentration up to 0.01-2.5 mg/ml, did not affected to the cell viability comparing to the negative control (all $p>$ 0.05, Fig. 4). The concentration of AC over than 2.5 mg/ml was limited for cytotoxicity test according to the colour of AC interfere MTT staining to the viable cells.

Freeze-dried powders of CT and CC were found to be very soluble in water. After forming complex, we observed that in the cytotoxicity test, AC3 was hardly dissolved in culture medium (pH7). Thus, to aid dissolution of AC for cytotoxicity study, solubility of the AC was carried out in 9 types of solvents as shown in Table IV. AC3, however, was slightly soluble in water. This is also the case with alcohol and organic solvents. It is the crystalline-like conformation of molecules of anthocyanins that changes the physicochemical properties of the anthocyanins. This influences the use of solvents in the cell studies, thus increasing the cytotoxicity of AC was limited according to the solubility of AC. Moreover, increasing the concentration of AC may affect the colour and provide a false positive in %viability due to absorption interference at 550 nm in the MTT viability assay.

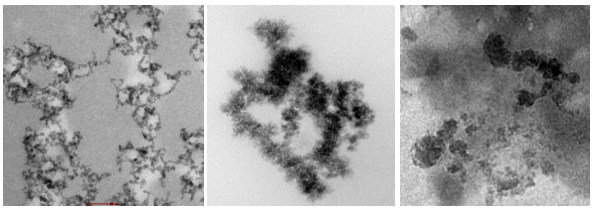
TABLE IV
SOLUBILITY OF ANTHOCYANIN COMPLEX

Solute	Solubility(mg/ml)	Solubility description (based on USP)
DI water pH 1	5.0	Slightly soluble
DI water pH 7	3.0	Slightly soluble
DI water pH 12	5.4	Slightly soluble
Methanol	>0.5	Very slightly soluble
Ethanol	>0.5	Very slightly
Hexane	insoluble	Insoluble
Chloroform	insoluble	Insoluble
DMSO	4.8	Slightly soluble
PEG400	>1.0	Very slightly soluble



(a) AC (b) CC (c) CT

Fig. 5 Scanning electron microscopic (SEM) photograph of anthocyanin complex (a), *Zea mays* L. *ceritina* Kulesh. (CC) (b) and *Clitoria ternatea* L. (CT) (c) (25,000×)



(a) AC (b) CC (c) CT

Fig. 6 Transmission electron microscopic (TEM) photographs of anthocyanin complex (AC) (a), *Zea mays* L. *ceritina* Kulesh.(CC) (b) and *Clitoria ternatea* L. (CT) (c) (25,000×)

The particle size of AC may also affect to the cells viability. Thus, the size of AC3 was investigated by laser diffraction using the Zetasizer. AC was shown to be nano-size range 100-

400 nm, but the size was increased after 2 h which representing the aggregation.

SEM photographs reveal the particles of CC, CT, and AC3 to be amorphous- and crystalline-like. The size of AC3 was between 50-200 nm (Fig. 5). TEM photographs show some improved orientation of molecular dispersions of AC than CC and CT (Fig. 6). These photographs confirmed the nano-size of AC3 which emphasized the safety after forming complex that within this range, AC3 could penetrate into the cells, but did not affect or caused cytotoxicity.

IV. Conclusion

A simplified innovative product composed of anthocyanins extracted from 2 natural resources, one of which being agricultural wastes which has not been previously explored by this method. The complexation of AC observed by UV-Vis spectroscopy and FTIR analysis revealed the changes in the wavelength of UV absorption and functional group of FTIR spectrum. AC was shown to be crystal like nano-size particles with no cytotoxic effect to human gingival epithelium cells (HGEPp0.5) and human gingival fibroblasts (HGF). Thus, this information of anthocyanin complex characterization and physicochemical properties could be used for further development and become a potential candidate to be used for the oral product in the future.

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